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(54) Title: VIRAL PARTICLES WITH EXOGENOUS INTERNAL EPITOPES

Insertions in the N-terminus of CPMV VP-S

pCP26:
CAAGGACCTGTTTGTGCTGAAGCCTCAGATGTGTATAGCCCATGTATGATAGCTAGCACTCCTCCTGC
GTTCCTGGACAAACACGACTTCGGAGTCTACACATATCGGGTACATACTATCGATCGTGAGGAGGACG
Q G P V C A E A S D V Y S P C M I A S T P P A

With NheI/Eco0109I:
CAAG
GTTCCTG

CTAGCACTCCTCCTGC
GTGAGGAGGACG

Inserts:
GACCTGTTTGTGCTGAAGCCTCAGATGTGTAT -epitope- TATAGCCCATGTATGATAG
GACAAACACGACTTCGGAGTCTACACATA ATATCGGGTACATACTATCGATC

duplication of y_{11}

(57) Abstract: The present invention relates to the expression of peptides on viral particles, and more particularly to the expression of peptides on the interior of the viral capsid. Methods are described for modifying viruses so that exogenous epitopes are expressed on the interior of the viral capsid. Viruses that can be modified include (+) stranded RNA viruses, especially plant (+) stranded RNA viruses such as the cowpea mosaic virus. Internal expression is especially useful for the expression of hydrophobic epitopes. The modified viral particles also find use as vaccines and as such are capable of eliciting an immune response.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

VIRAL PARTICLES WITH EXOGENOUS INTERNAL EPITOPES

FIELD OF THE INVENTION

The present invention relates to the expression of peptides on viral particles, and more particularly to the expression of peptides on the interior of the viral capsid.

BACKGROUND

Vaccines are one of the greatest achievements of biomedical science and public health. At the beginning of the 20th century, infectious diseases were widely prevalent in the United States and exacted an enormous toll on the population. For example, in 1900, 21,064 smallpox cases were reported, and 894 patients died. In 1920, 469,924 measles cases were reported, and 7575 patients died; 147,991 diphtheria cases were reported, and 13,170 patients died. In 1922, 107,473 pertussis cases were reported, and 5099 patients died. These diseases have largely been eliminated in the United States.

Despite this success, more than 5 million infants world-wide die every year from diseases that could be avoided with existing vaccines. However, many of the current vaccines must be refrigerated which makes their distribution in developing countries difficult. Furthermore, vaccines are either nonexistent or not available for diseases associated with significant rates of morbidity or mortality. For example, more than 250 million people are chronically infected with hepatitis B virus, malaria causes 1-2 million deaths each year; diarrheal diseases (for example, infections caused by rotovirus, *Shigella* sp., *Vibrio cholera*, and toxin producing *E. coli*) annually kill more an estimated 4-5 million people.

The Center for Disease Control has identified several factors for achieving the full potential of vaccines (www.cdc.gov/epo/mmwr/preview/mmwrhtml/00056803.htm). These suggestions include pursuing new approaches to vaccine delivery and administration. One new approach is the development of vaccines that stimulate the two types of immune responses: humoral responses mediated by B cells and cellular responses mediated by helper T-cells.

However, attempts to create vaccines that stimulate both humoral and cellular immune responses or preferentially induce a cellular immune response have met with difficulty. For example, synthetic peptide vaccines and recombinant protein vaccines are often poorly immunogenic and tend to induce humoral responses and not to induce cellular

immune responses. DNA vaccines can induce both humoral and cellular immune responses. However, questions remain as to what the consequences of long-term antigen expression will be.

Accordingly, what is needed in the art are improved delivery mechanisms for vaccines. In particular, the delivery mechanism should be useful inducing both cellular and humoral immune responses.

SUMMARY OF THE INVENTION

The present invention relates to the expression of peptides on viral particles, and more particularly to the expression of peptides on the interior or the viral capsid. In some embodiments, the present invention provides a compound comprising a chimeric viral particle having a capsid, wherein the capsid has an interior side and an exterior side, the capsid comprising at least one exogenous peptide on said interior side of the capsid. In some preferred embodiments, the viral particle is capable of assembly in a host cell or tissue. In some embodiments, the viral particle is icosahedral. In some preferred embodiments, the viral particle is a comovirus. In some particularly preferred embodiments, the viral particle is cowpea mosaic virus. In some embodiments, the exogenous peptide is inserted in a coat protein of the viral particle. In some preferred embodiments, the exogenous peptide has 5 to 20 amino acids. In other preferred embodiments, the exogenous peptide is inserted at a point from 5 to 20 amino acids from the N-terminus of a coat protein such assembly of the viral particle is not precluded in a host cell. In some particularly preferred embodiments, the exogenous peptide is inserted in VP-S of cowpea mosaic virus between a tyrosine residue at position 11 and a duplicated tyrosine residue at position 12. In other particularly preferred embodiments, the exogenous peptide is inserted in VP-S of cowpea mosaic virus between a dipeptide comprising a valine residue at position 10 and a tyrosine residue at position 11 and a duplicated dipeptide comprising a valine residue at position 12 and a tyrosine residue at position 13. In still other preferred embodiments, the exogenous peptide is inserted in VP-S of cowpea mosaic virus between a valine residue at position 10 and a duplicated valine residue at position 11. In further embodiments, the viral particle does not contain nucleic acid.

In other embodiments, the exogenous peptide encodes an epitope recognizable by an animal immune system. In some preferred embodiments, the exogenous epitope is a cytotoxic T lymphocyte epitope. In particularly preferred embodiments, the exogenous peptide contains a cytotoxic T lymphocyte epitope with flanking amino acids derived from a naturally occurring source of the epitope. In some embodiments, the exogenous peptide is a T helper cell epitope. In some preferred embodiments, the exogenous peptide contains a T helper cell epitope with flanking amino acid sequences derived from a naturally occurring source of the epitope. In still other embodiments, the exogenous peptide is a B cell epitope. In further embodiments, the exogenous peptide contains a T helper cell epitope with flanking amino acid sequences derived from a naturally occurring source of the epitope.

In some embodiments, the chimeric viral particle contains a second exogenous peptide expressed on the outer surface of the viral capsid. In preferred embodiments, the second exogenous peptide is expressed on the outer surface of the viral capsid, wherein said peptide is inserted in the βC'-βC" loop of VP-S of cowpea mosaic virus. In other preferred embodiments the second exogenous peptide is expressed on the outer surface of the viral capsid, wherein said peptide is inserted in the βC-βC loop of VP-S of cowpea mosaic virus. In still other preferred embodiments the second exogenous peptide is expressed on the outer surface of the viral capsid, wherein said peptide is inserted in the βE-βA loop of VP-L of cowpea mosaic virus.

In other embodiments, the present invention provides a vaccine composition characterized in having an effective amount of a viral particle comprising a capsid having an interior side and an exterior side, said capsid comprising at least one exogenous peptide, wherein said exogenous peptide is on said interior side of said capsid. In still other embodiments the present invention provides a formulation which comprises as an active ingredient a viral particle comprising a capsid having an interior side and an exterior side, said capsid comprising at least one exogenous peptide, wherein said exogenous peptide is on said interior side of said capsid and an adjuvant.

In some embodiments, the present invention provides a compound comprising a viral coat protein, wherein said viral coat protein includes an exogenous peptide, said viral coat protein is configured so as to assemble into a viral capsid having an interior side and an exterior side, wherein said exogenous peptide is expressed on said interior side of said viral capsid.

In further embodiments, the present invention provides a process for preparing a viral particle comprising providing a host cell and nucleic acid encoding a viral particle, said viral particle comprising i) a viral coat protein, said viral coat protein comprising an interior side and an exterior side, and ii) an exogenous peptide, wherein said exogenous peptide is inserted on said interior side of said viral coat protein; transfecting said host cell with said nucleic acid so that viral particles are produced.

In still other embodiments, the present invention provides a method of inducing an immune response in an animal requiring such treatment which method comprises administering to an animal a viral particle comprising a capsid having an interior side and an exterior side, said capsid comprising at least one exogenous peptide, wherein said exogenous peptide is on said interior side of said capsid.

In still further embodiments, the present invention provides a product obtainable by the process comprising providing a host cell and nucleic acid encoding a viral particle, said viral particle comprising i) a viral coat protein, said viral coat protein comprising an interior side and an exterior side, and ii) an exogenous peptide, wherein said exogenous peptide is inserted on said interior side of said viral coat protein; transfecting said host cell with said nucleic acid so that viral particles are produced.

In some embodiments, the present invention provides a commercial package comprising a viral particle comprising a capsid having an interior side and an exterior side, said capsid comprising at least one exogenous peptide, wherein said exogenous peptide is on said interior side of said capsid as an active ingredient together with instructions for use thereof. In other embodiments, the present compound comprising a chimeric virus particle expressing an internal epitope as described herein in any of the examples.

In some embodiments, the present invention provides a vector comprising nucleic acid encoding a viral particle, the viral particle comprising i) a viral coat protein comprising an interior side and an exterior side, and ii) an exogenous peptide, wherein said exogenous peptide is inserted on the interior side of the viral coat protein. The present invention is not limited to any particular type of vector. Indeed, a variety of vectors are contemplated, including, but not limited to RNA vectors (*for example*, nucleic acid encoding a (+) stranded RNA virus) or DNA vectors (*for example*, plasmid DNA encoding a (+) stranded RNA virus). Likewise, the present invention is not limited to any particular viral particle. Indeed, a variety of viral particles are contemplated, including, but not limited to, rod-shaped viral

particles and icosohedral-shaped viral particles. The present invention is not limited to any particular plant (+) stranded virus. Indeed, a variety of plant (+) stranded RNA viruses find use in the present invention. In some preferred embodiments, the plant (+) stranded RNA virus is a comovirus. In particularly preferred embodiments, the plant (+) stranded RNA virus is cow pea mosaic virus.

In some embodiments, the coat protein is derived from a (+) stranded RNA virus. The present invention is not limited to coat proteins from any particular (+) stranded RNA virus. Indeed, coat proteins from a variety of (+) stranded RNA viruses are contemplated. In some preferred embodiments, the coat protein is from a plant (+) stranded RNA virus. The present invention is not limited to insertion of the exogenous peptide at any particular location. Indeed, insertion at a variety of locations is contemplated. In some embodiments, the viral coat protein has an N-terminus, and the exogenous peptide is inserted at a position from 5 to 20 amino acids from the N-terminus so that assembly of said viral coat protein is not precluded. In some preferred embodiments, the viral coat protein is VP-S of cow pea mosaic virus and the exogenous peptide is inserted between a tyrosine residue at position 11 of the VP-S and a duplicated tyrosine residue engineered at position 12 of the VP-S. In other preferred embodiments, the viral coat protein is VP-S of cow pea mosaic virus and the exogenous peptide is inserted between a dipeptide comprising a valine residue at position 10 and a tyrosine residue at position 11 of the VP-S, and a duplicated dipeptide comprising a valine residue engineered at position 12 and a tyrosine residue engineered at position 13 of the VP-S.

The present invention is not limited to exogenous peptides of any particular type. Indeed, a variety of exogenous peptides can be expressed in the vectors of the present invention. In some embodiments, the exogenous peptide is hydrophobic. In other embodiments, the exogenous peptide is a cytotoxic T lymphocyte epitope. In further embodiments, the exogenous peptide is a helper T cell epitope. In still other embodiments, the exogenous peptide is a B cell epitope.

The present invention is not limited to vectors encoding only a single exogenous peptide. Indeed, the present invention contemplates that more than one exogenous peptide can be expressed from the vectors. In some embodiments, the viral coat protein further comprises a second exogenous peptide. In preferred embodiments, the second exogenous peptide is inserted on the exterior side of said viral coat protein. In some particularly

preferred embodiments, the viral coat protein is VP-S having a βC'-βC" loop and the second exogenous peptide is inserted in said βC'-βC" loop. In other preferred embodiments, the viral coat protein is VP-L having a βE-βA loop and the second exogenous peptide is inserted in said βE-βA loop.

The vectors of the present invention also include other components, such as regulatory elements. In some embodiments, the nucleic acid further encodes a promoter operably linked to the nucleic acid encoding a viral particle. The present invention is not limited to any particular promoter. Indeed, a variety of promoters are contemplated, including, but not limited to tissue specific plant promoters and constitutive plant promoters.

In other embodiments, the present invention provides methods comprising providing i)the vector described above and ii) host cells; and b) transfecting the host cells with the vector to produce transfected host cells under conditions such that the transfected host cells express the viral particle. The present invention is not limited to the transfection of any particular host cells. Indeed, the transfection of a variety of host cells is contemplated, including, but not limited to, host cells selected from the group consisting of cells in planta, plant tissue culture cells, plant protoplasts, and cells in plant tissue. In still further embodiments, the present invention encompasses host cells produced by these methods.

In some embodiments, the present invention provides methods comprising providing a plant transfected with the vector described above and growing the plant under conditions such that the viral particle is produced. In some preferred embodiments, the methods further comprise the step of purifying the viral particles from the plant.

In further embodiments, the present invention provides compositions comprising a nucleic acid encoding a viral coat protein comprising an exogenous peptide, the viral coat protein is configured so as to assemble into a viral capsid having an interior side and an exterior side, wherein the exogenous peptide is expressed on the interior side of the viral capsid. The present invention is not limited to any particular type of nucleic acid. Indeed, a variety of nucleic acids are contemplated, including, but not limited to RNA (*for example*, nucleic acid encoding a (+) stranded RNA virus) or DNA (*for example*, plasmid DNA encoding a (+) stranded RNA virus). Likewise, the present invention is not limited to any particular viral particle. Indeed, a variety of viral particles are contemplated, including, but not limited to, rod-shaped viral particles and icosohedral-shaped viral particles. The present invention is not limited to any particular plant (+) stranded virus. Indeed, a variety of plant

(+) stranded RNA viruses find use in the present invention. In some preferred embodiments, the plant (+) stranded RNA virus is a comovirus. In particularly preferred embodiments, the plant (+) stranded RNA virus is cow pea mosaic virus.

In some embodiments, the coat protein is derived from a (+) stranded RNA virus. The present invention is not limited to coat proteins from any particular (+) stranded RNA virus. Indeed, coat proteins from a variety of (+) stranded RNA viruses are contemplated. In some preferred embodiments, the coat protein is from a plant (+) stranded RNA virus. The present invention is not limited to insertion of the exogenous peptide at any particular location. Indeed, insertion at a variety of locations is contemplated. In some embodiments, the viral coat protein has an N-terminus, and the exogenous peptide is inserted at a position from 5 to 20 amino acids from the N-terminus so that assembly of said viral coat protein is not precluded. In some preferred embodiments, the viral coat protein is VP-S of cow pea mosaic virus and the exogenous peptide is inserted between a tyrosine residue at position 11 of the VP-S and a duplicated tyrosine residue engineered at position 12 of the VP-S. In other preferred embodiments, the viral coat protein is VP-S of cow pea mosaic virus and the exogenous peptide is inserted between a dipeptide comprising a valine residue at position 10 and a tyrosine residue at position 11 of the VP-S, and a duplicated dipeptide comprising a valine residue engineered at position 12 and a tyrosine residue engineered at position 13 of the VP-S.

The present invention is not limited to exogenous peptides of any particular type. Indeed, a variety of exogenous peptides can be expressed in the vectors of the present invention. In some embodiments, the exogenous peptide is hydrophobic. In other embodiments, the exogenous peptide is a cytotoxic T lymphocyte epitope. In further embodiments, the exogenous peptide is a helper T cell epitope. In still other embodiments, the exogenous peptide is a B cell epitope.

The present invention is not limited to nucleic acids encoding only a single exogenous peptide. Indeed, the present invention contemplates that more than one exogenous peptide can be encoded by the nucleic acids. In some embodiments, the viral coat protein further comprises a second exogenous peptide. In preferred embodiments, the second exogenous peptide is inserted on the exterior side of the viral coat protein. In some particularly preferred embodiments, the viral coat protein is VP-S having a βC'-βC" loop and the second exogenous peptide is inserted in said βC'-βC" loop. In other preferred embodiments, the

viral coat protein is VP-L having a βE-βA loop and the second exogenous peptide is inserted in said βE-βA loop.

The nucleic acids of the present invention also include other components, such as regulatory elements. In some embodiments, the nucleic acids further encode a promoter operably linked to the nucleic acid encoding a viral particle. The present invention is not limited to any particular promoter. Indeed, a variety of promoters are contemplated, including, but not limited to tissue specific plant promoters and constitutive plant promoters.

In still further embodiments, the present invention provides viral particles comprising a capsid having an interior side and an exterior side, the capsid comprising at least one exogenous peptide, wherein the exogenous peptide is on the interior side of the capsid. The present invention is not limited to any particular viral particle. Indeed, as described above, the present invention encompasses a wide variety of viral particles. In some embodiments, the present invention provides a plant expressing the viral particles. In further embodiments, the present invention provides fruit, leaves, tubers, stems or purified viral particles isolated from the plant.

In other embodiments, the present invention provides methods for inducing an immune response comprising providing i) viral particles (described above) comprising a plurality of coat proteins having an interior side and an exterior side, the coat proteins comprising an exogenous peptide, wherein the exogenous peptide is on the interior side of the coat proteins; and ii) a subject; and b) exposing the subject to the viral particle under conditions such that the subject develops an immune response to the exogenous peptide. In some preferred embodiments, the viral particles are provided from a plant source.

In still further embodiments, the present invention provides vectors comprising nucleic acid encoding a viral coat protein sequence having inserted therein an exogenous peptide sequence, the viral coat protein comprising a second site mutation such that the viral coat protein is capable of being assembled into a viral capsid. The present invention not limited to any particular second site mutations. Indeed, a variety of second site mutation are contemplated, including, but not limited to, second site mutations in both the VP-S and VP-L of cow pea mosaic virus. In particularly preferred embodiments, the second site mutation is selected from the group consisting of F91S in VP-S, F180L in VP-S, M177V in VP-S, I124V in VP-S, R2102K in VP-L, I2045M in VP-L, M177T in VP-S, A2092T in VP-L, G80D in VP-S.

In some embodiments, the present invention provides methods for inducing second site mutations in a viral coat protein comprising providing i) a vector comprising nucleic acid encoding a viral particle, the viral particle comprising a viral coat protein, the viral coat protein comprising an interior side and an exterior side, and ii) a foreign peptide, wherein the foreign peptide is inserted on the interior side of the viral coat protein and ii) a first host plant; infecting the first host plant with the vector so that the viral particle is expressed; monitoring the first host plant until late lesions appear in directly infected leaves; isolating viral particles from the late lesions to provide isolated viral particles; and inoculating a second host plant to obtain a secondary infection. In some embodiments, the methods further comprise the step of monitoring the second host plant for the appearance of systemic symptoms. In some preferred embodiments, the systemic symptoms appear in a time frame comparable to that of plant infected with control viral particles. In other embodiments, the methods further comprise the steps of The method of Claim 84, further comprising the steps of isolating viral particles from the systemic lesions, the viral particles comprising a genome; and sequencing the genome of the viral particles. In some embodiments, the present invention provides the viral particles produced by the method.

In some embodiments, the present invention provides a vaccine composition comprising a viral particle comprising a capsid having an interior side and an exterior side, the capsid comprising at least one exogenous peptide, wherein the exogenous peptide is on the interior side of the capsid. In further embodiments, the present invention provides methods of inducing a humoral immune response which comprises administering to an animal the vaccine composition.

In still other embodiments, the present invention provides methods of enhancing an immune response to a B cell epitope comprising providing i) a modified viral particle comprising a B cell epitope and an internal CTL epitope in an immunogenic complex; and ii) an animal; and administering the modified viral particle to the animal. The present invention is not limited to and particular CTL epitope. Indeed, a variety of CTL epitopes are contemplated including, but not limited to, a 2F10 peptide mimotope of the class "a" determinant of the hepatitis B virus surface antigen.

The present invention also provides methods of enhancing an immune response to a B cell epitope comprising providing i) a modified viral particle comprising a B cell epitope and an internal CTL epitope in an antigenic complex; and an animal; and b) administering the

modified viral particle to the animal. The present invention is not limited to and particular CTL epitope. Indeed, a variety of CTL epitopes are contemplated including, but not limited to, a 2F10 peptide mimotope of the class "a" determinant of the hepatitis B virus surface antigen.

In some embodiments, the present invention provides methods of enhancing an immune response to a B cell epitope comprising providing i) a modified viral particle comprising a B cell epitope and an internal helper T cell epitope in an immunogenic complex; and ii) an animal; and administering the modified viral particle to said animal. The present invention is not limited to any particular T cell epitope. Indeed, a variety of T cell epitopes are contemplated, including, but not limited to the universal T helper epitope of tetanus toxoid.

In further embodiments, the present invention provides methods of enhancing an immune response to a B cell epitope comprising providing i) a modified viral particle comprising a B cell epitope and an internal helper T cell epitope in an antigenic complex; and ii) an animal; and administering the modified viral particle to the animal. The present invention is not limited to any particular T cell epitope. Indeed, a variety of T cell epitopes are contemplated, including, but not limited to the universal T helper epitope of tetanus toxoid.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the sequence of the N-terminus of the VP-S protein of CPMV and illustrates where foreign peptides can be inserted.

Figure 2 shows the results of a CTL assay for mice vaccinated with chimeric virus particles according to one embodiment of the invention (see Example 6); the chromium release from target cells charged with the target peptide derived from LCMV or uncharged cells is measured using a BetaMax workstation.

Figure 3 shows a restriction map of the vector pCP26 used in the majority of genetic constructions disclosed.

DESCRIPTION OF THE INVENTION

The present invention relates to the expression of peptides on viral particles, and more particularly to the expression of peptides on the interior or the viral capsid. The use of

chimeric viral particles (CVP's) as vaccines represents an attractive strategy for improving vaccine storage and delivery. The use of CVPs is described in U.S. Pat. Nos. 5,874,087 and 5,958,422 (each of which incorporated herein by reference). These patents describe the development of vectors encoding modified Cow Pea Mosaic Virus (CPMV) genomes containing peptide inserts in the coat protein. The vectors are useful for the production of viral particles in which the peptide is presented on the surface of the viral particle.

After the definitions, viral particle expression systems, peptide inserts and sites for insertion, and uses for modified viral particles are described.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The term "viral particle" as used herein refers to the fully or partially assembled capsid of a virus. A viral particle may or may not contain nucleic acid.

The term "viral capsid" as used herein refers to the protein coat that surrounds the viral nucleic acid in a wild-type virus. Viral capsids have interior surfaces and exterior surfaces. The interior surface of a viral capsid is the surface that is normally exposed to the viral nucleic acid. The exterior surface of a viral capsid is the surface that is generally exposed to the environment.

The term "viral coat protein" as used herein refers to a protein that interacts with other proteins to assemble and form part of the viral capsid. Examples of viral coat proteins include, but are not limited to, the VP-S and VP-L coat proteins of cowpea mosaic virus. The interior side of a viral coat protein is the portion of the coat protein that is exposed on the interior surface of the viral capsid. The exterior side of a viral coat protein is the portion of the coat protein that exposed on the exterior surface of the viral capsid.

The term "(+) stranded RNA virus" as used herein refers to a virus having an RNA genome, wherein the isolated RNA is directly infectious when introduced to an appropriate host. (+) stranded RNA viruses are known to infect a variety of animal, fungus, and plant hosts. Examples of (+) stranded RNA viruses include, but are not limited to, Picornoviruses (*for example*, polioviruses), and viruses from the following families: *Caulimoviridae*, *Bromoviridae*, *Comoviridae*, *Geminiviridae*, *Reoviridae*, *Partitiviridae*, *Sequiviridae*, *Tobamoviruses*, and *Tombusviridae*.

The term "symptoms" when used in reference to plant virus infection refers to the appearance of indicators of viral infection in the plant. These indicators both local lesions and systemic symptoms. Local lesions such as necrotic lesions, chlorotic lesions, and ringspot occur at or near the site of infection. Systemic symptoms appear throughout the plant and include mosaic patterns, mottled patterns, dwarfing, yellowing and necrosis.

The term "systemic infection" as used herein refers to viral infections that spread from the site of initial infection. In plants, systemic infection occurs when the viruses move from infected cells into the vasculature (*for example*, phloem) of the plant. In many viruses, this movement is mediated by a movement protein which modifies the plasmadesmata.

The term "icosahedral," when used in reference to viral capsid or viral particle refers to a capsid exhibiting general icosohedral symmetry: 5-fold rotational symmetry through each of 12 apexes, 3-fold rotational symmetry about an axis through the center of each of 20 triangular faces, and 3-fold rotational symmetry about an axis through the center of each of thirty edges. Icosohedrons are comprised of 60 identical building units (which may comprise more than one subunit) or multiples of 60 identical building units. The interunit contacts are not precisely identical throughout the capsid; however, all interunit bonding involves the same general type of contact so that the interunit bonds can be described as quasi-equivalent. It is contemplated that some icosohedral viruses, especially large icosohedral viruses (*for example*, adenoviruses) may deviate from the structural and geometrical criteria observed by smaller icosohedral viruses. Examples of icosohedral viruses include, but are not limited to, polioviruses, adenoviruses, and viruses from the following families: *Caulimoviridae*, *Bromoviridae*, *Comoviridae*, *Geminiviridae*, *Reoviridae*, *Partitiviridae*, *Sequiviridae*, and *Tombusviridae*.

The term "epitope" as used herein refers to an antigenic determinant, which is any region of a macromolecule with the ability or potential to elicit, and combine with, specific antibody (*that is*, capable of binding to a specific immunoglobulin or T-cell receptor).

The term "hydrophobic," when used in reference to a peptide or epitope, refers to a peptide or epitope having about 20 percent or greater hydrophobic amino acids residues (*for example*, alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, and methionine).

The term "cytotoxic T-lymphocyte epitope" as used herein refers to an epitope that is capable of recognition by a cytotoxic T-lymphocyte.

The term "helper T-cell epitope" as used herein refers to an epitope that is capable of recognition by a helper T-cell.

The term "B-cell epitope" as used herein refers to an epitope that is capable of recognition by a B-cell.

The term "immune response" as used herein refers to an animal's reaction mediated by the immune system to an antigen or immunogen and may be characterized by the production of antibodies and/or the stimulation of cell-mediated or immune tolerance.

The term "antigenic complex" as used herein refers to complex containing at least one epitope that is capable of combining with an immunoglobulin or cell-surface receptor.

The term "immune complex" as used herein refers to a complex containing at least one epitope that is capable of eliciting a humoral and/or cell-mediated immune response.

The term "mimotope" as used herein refers to a peptide sufficiently structurally similar to an epitope to induce an immune reaction against that epitope even though the two sequences share no homology or similarity at the amino acid level (in the case of a peptide mimotope), or, where in the case where the mimotope represents a structural configuration adopted by a non-proteinaceous molecule such as a carbohydrate, the mimotope is capable of reacting with immune molecules directed against that non-proteinaceous epitope.

As used herein, the term "immunoglobulin" refers to the secreted product of plasma cell (*for example*, activated B-cell) comprising two heavy chain polypeptides complexed with two light chain polypeptides which together make a binding site for proteins.

As used herein, the term "MHC Class I-major histocompatibility group I proteins" refers to proteins encoded by the major histocompatibility group genes and which are implicated in the effective presentation of antigens on CD8+ T lymphocytes.

As used herein, the term "MHC Class II- major histocompatibility group II proteins" refers to proteins encoded by the major histocompatibility group genes and which are implicated in the effective presentation of antigens on CD4+ T lymphocytes.

The term "plant" as used herein refers to a plurality of plant cells which are largely differentiated into a structure that is present at any stage of a plant's development. Such structures include, but are not limited to, a fruit, shoot, stem, leaf, flower petal, *etc.* The term "plant tissue" includes differentiated and undifferentiated tissues of plants including, but not limited to, roots, shoots, leaves, pollen, seeds, tumor tissue and various types of cells in

culture (*for example*, single cells, protoplasts, embryos, callus, *etc.*). Plant tissue may be *in planta*, in organ culture, tissue culture, or cell culture.

The term "protoplast" as used herein refers to isolated plant cells in which the cell walls have been removed. In general, protoplasts are produced in accordance with conventional methods (*See, for example*, U.S. Pat. Nos. 4,743,548; 4,677,066, 5,149,645; and 5,508,184; all of which are incorporated herein by reference). Plant tissue may be dispersed in an appropriate medium having an appropriate osmotic potential (*for example*, 3 to 8 wt. percent of a sugar polyol) and one or more polysaccharide hydrolases (*for example*, pectinase, cellulase, etc.), and the cell wall degradation allowed to proceed for a sufficient time to provide protoplasts. After filtration the protoplasts may be isolated by centrifugation and may then be resuspended for subsequent treatment or use. Regeneration of protoplasts kept in culture to whole plants is performed by methods known in the art (*See, for example*, Evans *et al.*, *Handbook of Plant Cell Culture*, 1: 124-176, MacMillan Publishing Co., New York [1983]; Binding, *Plant Protoplasts*, p. 21-37, CRC Press, Boca Raton [1985],) and Potrykus and Shillito, *Methods in Enzymology*, Vol. 118, Plant Molecular Biology, A. and H. Weissbach eds., Academic Press, Orlando [1986]).

The term "gene" as used herein, refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or protein precursor. The polypeptide can be encoded by a full-length coding sequence or by any portion of the coding sequence, as long as the desired protein activity is retained.

"Nucleoside", as used herein, refers to a compound consisting of a purine [guanine (G) or adenine (A)] or pyrimidine [thymine (T), uridine (U), or cytidine (C)] base covalently linked to a pentose, whereas "nucleotide" refers to a nucleoside phosphorylated at one of its pentose hydroxyl groups.

A "nucleic acid", as used herein, is a covalently linked sequence of nucleotides in which the 3' position of the pentose of one nucleotide is joined by a phosphodiester group to the 5' position of the pentose of the next, and in which the nucleotide residues (bases) are linked in specific sequence; *that is*, a linear order of nucleotides. A "polynucleotide", as used herein, is a nucleic acid containing a sequence that is greater than about 100 nucleotides in length. An "oligonucleotide", as used herein, is a short polynucleotide or a portion of a polynucleotide. An oligonucleotide typically contains a sequence of about two to about one hundred bases. The word "oligo" is sometimes used in place of the word "oligonucleotide".

Nucleic acid molecules are said to have a "5'-terminus" (5' end) and a "3'-terminus" (3' end) because nucleic acid phosphodiester linkages occur to the 5' carbon and 3' carbon of the pentose ring of the substituent mononucleotides. The end of a nucleic acid at which a new linkage would be to a 5' carbon is its 5' terminal nucleotide. The end of a nucleic acid at which a new linkage would be to a 3' carbon is its 3' terminal nucleotide. A terminal nucleotide, as used herein, is the nucleotide at the end position of the 3'- or 5'-terminus. DNA molecules are said to have "5' ends" and "3' ends" because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage. Therefore, an end of an oligonucleotides referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring.

As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide or polynucleotide, also may be said to have 5' and 3' ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements. This terminology reflects the fact that transcription proceeds in a 5' to 3' fashion along the DNA strand. Typically, promoter and enhancer elements that direct transcription of a linked gene are generally located 5' or upstream of the coding region. However, enhancer elements can exert their effect even when located 3' of the promoter element and the coding region. Transcription termination and polyadenylation signals are located 3' or downstream of the coding region.

The term "wild-type" when made in reference to a gene refers to a gene which has the characteristics of a gene isolated from a naturally occurring source. The term "wild-type" when made in reference to a gene product refers to a gene product which has the characteristics of a gene product isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" when made in reference to a gene or to a gene product refers, respectively, to a gene or to a gene product which displays modifications in sequence and/or functional properties (*that is*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by

the fact that they have altered characteristics when compared to the wild-type gene or gene product.

As used herein, the term "overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms. As used herein, the term "cosuppression" refers to the expression of a foreign gene which has substantial homology to an endogenous gene resulting in the suppression of expression of both the foreign and the endogenous gene. As used herein, the term "altered levels" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms.

The term "recombinant" when made in reference to a DNA molecule refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques. The term "recombinant" when made in reference to a protein or a polypeptide refers to a protein molecule which is expressed using a recombinant DNA molecule.

The term "nucleotide sequence of interest" refers to any nucleotide sequence, the manipulation of which may be deemed desirable for any reason (*for example*, confer improved qualities), by one of ordinary skill in the art. Such nucleotide sequences include, but are not limited to, coding sequences of structural genes (*for example*, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*), and non-coding regulatory sequences which do not encode an mRNA or protein product, (*for example*, promoter sequence, polyadenylation sequence, termination sequence, enhancer sequence, *etc.*).

As used herein the term "coding region" when used in reference to structural gene refers to the nucleotide sequences which encode the amino acids found in the nascent polypeptide as a result of translation of a mRNA molecule. Typically, the coding region is bounded on the 5' side by the nucleotide triplet "ATG" which encodes the initiator methionine and on the 3' side by a stop codon (*for example*, TAA, TAG, TGA). In some cases the coding region is also known to initiate by a nucleotide triplet "TTG".

As used herein, the terms "complementary" or "complementarity" when used in reference to polynucleotides refer to polynucleotides which are related by the base-pairing rules. For example, for the sequence 5'-AGT-3' is complementary to the sequence 5'-ACT-3'. Complementarity may be "partial," in which only some of the nucleic acids' bases are

matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods which depend upon binding between nucleic acids.

A "complement" of a nucleic acid sequence as used herein refers to a nucleotide sequence whose nucleic acids show total complementarity to the nucleic acids of the nucleic acid sequence.

The term "homology" when used in relation to nucleic acids refers to a degree of complementarity. There may be partial homology or complete homology (*that is*, identity). "Sequence identity" refers to a measure of relatedness between two or more nucleic acids or proteins, and is given as a percentage with reference to the total comparison length. The identity calculation takes into account those nucleotide or amino acid residues that are identical and in the same relative positions in their respective larger sequences. Calculations of identity may be performed by algorithms contained within computer programs such as "GAP" (Genetics Computer Group, Madison, Wis.) and "ALIGN" (DNAStar, Madison, Wis.).

A partially complementary sequence is one that at least partially inhibits (or competes with) a completely complementary sequence from hybridizing to a target nucleic acid is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (*that is*, the hybridization) of a sequence which is completely homologous to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (*that is*, selective) interaction. The absence of non-specific binding may be tested by the use of a second target which lacks even a partial degree of complementarity (*for example*, less than about 30 percent identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe which can hybridize to either or both strands of the double-stranded nucleic acid sequence under conditions of low stringency as described *infra*.

Low stringency conditions when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l $NaH_2PO_4 \cdot H_2O$ and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1 percent SDS, 5X Denhardt's reagent [50X Denhardt's contains per 500 ml: 5 g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V; Sigma)] and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 5X SSPE, 0.1 percent SDS at 42°C when a probe of about 500 nucleotides in length is employed.

High stringency conditions when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l $NaH_2PO_4 \cdot H_2O$ and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5 percent SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1X SSPE, 1.0 percent SDS at 42°C when a probe of about 500 nucleotides in length is employed.

When used in reference to nucleic acid hybridization the art knows well that numerous equivalent conditions may be employed to comprise either low or high stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other components (*for example*, the presence or absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of either low or high stringency hybridization different from, but equivalent to, the above listed conditions.

"Stringency" when used in reference to nucleic acid hybridization typically occurs in a range from about T_m-5°C (5°C below the T_m of the probe) to about 20°C to 25°C below T_m. As will be understood by those of skill in the art, a stringent hybridization can be used to identify or detect identical polynucleotide sequences or to identify or detect similar or related polynucleotide sequences. Under "stringent conditions" a nucleic acid sequence of interest will hybridize to its exact complement and closely related sequences.

Polypeptide molecules are said to have an "amino terminus" (N-terminus) and a "carboxy terminus" (C-terminus) because peptide linkages occur between the backbone amino group of a first amino acid residue and the backbone carboxyl group of a second amino acid residue. Typically, the terminus of a polypeptide at which a new linkage would be to the carboxy-terminus of the growing polypeptide chain, and polypeptide sequences are written from left to right beginning at the amino terminus.

As used herein, the terms "exogenous peptide" or "foreign peptide" refers to a peptide that is not in its natural environment (*that is*, has been altered by the hand of man). For example, an exogenous peptide gene includes a peptide that has been inserted into another polypeptide or added or fused to a polypeptide.

As used herein in reference to an amino acid sequence or a protein, the term "portion" (as in "a portion of an amino acid sequence") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (*for example*, viral coat protein) joined to an exogenous protein fragment (*for example*, a hydrophobic epitope).

The term "isolated" when used in relation to a nucleic acid, as in "an isolated nucleic acid sequence" refers to a nucleic acid sequence that is identified and separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is nucleic acid present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids are nucleic acids such as DNA and RNA which are found in the state they exist in nature. For example, a given DNA sequence (*for example*, a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs which encode a multitude of proteins. However, an isolated nucleic acid sequence comprising SEQ ID NO:X includes, by way of example, such nucleic acid sequences in cells which ordinarily contain SEQ ID NO:X where the nucleic acid sequence is in a chromosomal or extrachromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid sequence may be present in single-stranded or double-stranded form. When an isolated nucleic acid

sequence is to be utilized to express a protein; the nucleic acid sequence will contain at a minimum at least a portion of the sense or coding strand (*that is*, the nucleic acid sequence may be single-stranded). Alternatively, it may contain both the sense and anti-sense strands (*that is*, the nucleic acid sequence may be double-stranded).

As used herein, the term "purified" refers to molecules or aggregations of molecules (*for example*, viral particles), either nucleic or amino acid sequences, that are removed from their natural environment, isolated or separated. An "isolated nucleic acid sequence" is therefore a purified nucleic acid sequence. "Substantially purified" molecules are at least 60 percent free, preferably at least 75 free, and more preferably at least 90 percent free from other components with which they are naturally associated.

As used herein, the terms "vector" and "vehicle" are used interchangeably in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. Vectors may include plasmids, bacteriophages, viruses, cosmids, and the like.

The term "expression vector" or "expression cassette" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

The terms "targeting vector" or "targeting construct" refer to oligonucleotide sequences comprising a gene of interest flanked on either side by a recognition sequence which is capable of homologous recombination of the DNA sequence located between the flanking recognition sequences.

The terms "in operable combination", "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

Transcriptional control signals in eukaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription (Maniatis, *et al.*, Science

236:1237, 1987). Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect, mammalian and plant cells. Promoter and enhancer elements have also been isolated from viruses and analogous control elements, such as promoters, are also found in prokaryotes. The selection of a particular promoter and enhancer depends on the cell type used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types (*for review, see* Voss, *et al.*, Trends Biochem. Sci., 11:287, 1986; and Maniatis, *et al.*, *supra* 1987).

The terms "promoter element," "promoter," or "promoter sequence" as used herein, refer to a DNA sequence that is located at the 5' end (*that is* precedes) the protein coding region of a DNA polymer. The location of most promoters known in nature precedes the transcribed region. The promoter functions as a switch, activating the expression of a gene. If the gene is activated, it is said to be transcribed, or participating in transcription. Transcription involves the synthesis of mRNA from the gene. The promoter, therefore, serves as a transcriptional regulatory element and also provides a site for initiation of transcription of the gene into mRNA.

Promoters may be tissue specific or cell specific. The term "tissue specific" as it applies to a promoter refers to a promoter that is capable of directing selective expression of a nucleotide sequence of interest to a specific type of tissue (*for example*, seeds) in the relative absence of expression of the same nucleotide sequence of interest in a different type of tissue (*for example*, leaves). Tissue specificity of a promoter may be evaluated by, for example, operably linking a reporter gene to the promoter sequence to generate a reporter construct, introducing the reporter construct into the genome of a plant such that the reporter construct is integrated into every tissue of the resulting transgenic plant, and detecting the expression of the reporter gene (*for example*, detecting mRNA, protein, or the activity of a protein encoded by the reporter gene) in different tissues of the transgenic plant. The detection of a greater level of expression of the reporter gene in one or more tissues relative to the level of expression of the reporter gene in other tissues shows that the promoter is specific for the tissues in which greater levels of expression are detected. The term "cell type specific" as applied to a promoter refers to a promoter which is capable of directing selective expression of a nucleotide sequence of interest in a specific type of cell in the relative absence of expression of the same nucleotide sequence of interest in a different

type of cell within the same tissue. The term "cell type specific" when applied to a promoter also means a promoter capable of promoting selective expression of a nucleotide sequence of interest in a region within a single tissue. Cell type specificity of a promoter may be assessed using methods well known in the art, *for example*, immunohistochemical staining. Briefly, tissue sections are embedded in paraffin, and paraffin sections are reacted with a primary antibody which is specific for the polypeptide product encoded by the nucleotide sequence of interest whose expression is controlled by the promoter. A labeled (*for example*, peroxidase conjugated) secondary antibody which is specific for the primary antibody is allowed to bind to the sectioned tissue and specific binding detected (*for example*, with avidin/biotin) by microscopy.

Promoters may be constitutive or regulatable. The term "constitutive" when made in reference to a promoter means that the promoter is capable of directing transcription of an operably linked nucleic acid sequence in the absence of a stimulus (*for example*, heat shock, chemicals, light, *etc.*). Typically, constitutive promoters are capable of directing expression of a transgene in substantially any cell and any tissue. Exemplary constitutive plant promoters include, but are not limited to SD Cauliflower Mosaic Virus (CaMV SD; *see for example*, U.S. Pat. No. 5,352,605, incorporated herein by reference), mannopine synthase, octopine synthase (ocs), superpromoter (*see for example*, WO 95/14098), and *ubi3* (*see for example*, Garbarino and Belknap, Plant Mol. Biol. 24:119-127 [1994]) promoters. Such promoters have been used successfully to direct the expression of heterologous nucleic acid sequences in transformed plant tissue.

In contrast, a "regulatable" promoter is one which is capable of directing a level of transcription of an operably linked nuclei acid sequence in the presence of a stimulus (*for example*, heat shock, chemicals, light, *etc.*) which is different from the level of transcription of the operably linked nucleic acid sequence in the absence of the stimulus.

As used herein, the term "regulatory element" refers to a genetic element that controls some aspect of the expression of nucleic acid sequence(s). For example, a promoter is a regulatory element that facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements are splicing signals, polyadenylation signals, termination signals, etc.

The enhancer and/or promoter may be "endogenous" or "exogenous" or "heterologous." An "endogenous" enhancer or promoter is one that is naturally linked with a

given gene in the genome. An "exogenous" or "heterologous" enhancer or promoter is one that is placed in juxtaposition to a gene by means of genetic manipulation (*that is*, molecular biological techniques) such that transcription of the gene is directed by the linked enhancer or promoter. For example, an endogenous promoter in operable combination with a first gene can be isolated, removed, and placed in operable combination with a second gene, thereby making it a "heterologous promoter" in operable combination with the second gene. A variety of such combinations are contemplated (*for example*, the first and second genes can be from the same species, or from different species.

The presence of "splicing signals" on an expression vector often results in higher levels of expression of the recombinant transcript in eukaryotic host cells. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site (Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York [1989] pp. 16.7-16.8). A commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40.

Efficient expression of recombinant DNA sequences in eukaryotic cells requires expression of signals directing the efficient termination and polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly(A) site" or "poly(A) sequence" as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable, as transcripts lacking a poly(A) tail are unstable and are rapidly degraded. The poly(A) signal utilized in an expression vector may be "heterologous" or "endogenous." An endogenous poly(A) signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly(A) signal is one which has been isolated from one gene and positioned 3' to another gene. A commonly used heterologous poly(A) signal is the SV40 poly(A) signal. The SV40 poly(A) signal is contained on a 237 bp *Bam*HI/*Bcl*I restriction fragment and directs both termination and polyadenylation (Sambrook, *supra*, at 16.6-16.7).

The terms "infecting" and "infection" with a bacterium refer to co-incubation of a target biological sample, (*for example*, cell, tissue, *etc.*) with the bacterium under conditions such that nucleic acid sequences contained within the bacterium are introduced into one or more cells of the target biological sample.

The terms "bombarding, "bombardment," and "biolistic bombardment" refer to the process of accelerating particles towards a target biological sample (*for example*, cell, tissue, *etc.*) to effect wounding of the cell membrane of a cell in the target biological sample and/or entry of the particles into the target biological sample. Methods for biolistic bombardment are known in the art (*for example*, U.S. Patent No. 5,584,807, the contents of which are incorporated herein by reference), and are commercially available (*for example*, the helium gas-driven microprojectile accelerator (PDS-1000/He, BioRad).

The term "microwounding" when made in reference to plant tissue refers to the introduction of microscopic wounds in that tissue. Microwounding may be achieved by, for example, particle bombardment as described herein or by abrading the tissue.

The term "transfection" as used herein refers to the introduction of foreign DNA into eukaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics.

The term "transgenic" when used in reference to a cell refers to a cell which contains a transgene, or whose genome has been altered by the introduction of a transgene. The term "transgenic" when used in reference to a tissue or to a plant refers to a tissue or plant, respectively, which comprises one or more cells that contain a transgene, or whose genome has been altered by the introduction of a transgene. Transgenic cells, tissues and plants may be produced by several methods including the introduction of a "transgene" comprising nucleic acid (usually DNA) into a target cell or integration of the transgene into a chromosome of a target cell by way of human intervention, such as by the methods described herein.

The term "foreign gene" refers to any nucleic acid (*for example*, gene sequence) which is introduced into the genome of a cell by experimental manipulations and may include gene sequences found in that cell so long as the introduced gene contains some modification (*for example*, a point mutation, the presence of a selectable marker gene, *etc.*) relative to the naturally-occurring gene.

The term "transformation" as used herein refers to the introduction of a transgene into a cell. Transformation of a cell may be stable or transient. The term "transient transformation" or "transiently transformed" refers to the introduction of one or more

transgenes into a cell in the absence of integration of the transgene into the host cell's genome. Transient transformation may be detected by, for example, enzyme-linked immunosorbent assay (ELISA) which detects the presence of a polypeptide encoded by one or more of the transgenes. Alternatively, transient transformation may be detected by detecting the activity of the protein (*for example*, β-glucuronidase) encoded by the transgene. The term "transient transformant" refers to a cell which has transiently incorporated one or more transgenes. In contrast, the term "stable transformation" or "stably transformed" refers to the introduction and integration of one or more transgenes into the genome of a cell. Stable transformation of a cell may be detected by Southern blot hybridization of genomic DNA of the cell with nucleic acid sequences which are capable of binding to one or more of the transgenes. Alternatively, stable transformation of a cell may also be detected by the polymerase chain reaction of genomic DNA of the cell to amplify transgene sequences. The term "stable transformant" refers to a cell which has stably integrated one or more transgenes into the genomic DNA. Thus, a stable transformant is distinguished from a transient transformant in that, whereas genomic DNA from the stable transformant contains one or more transgenes, genomic DNA from the transient transformant does not contain a transgene.

A. Viral Particle Expression Systems

To produce modified virus particles in accordance with this invention the viral nucleic acid is modified by introducing a nucleotide sequence coding for the foreign peptide (*for example*, an animal virus antigen) at that part of the viral genome which codes for a portion of the coat protein exposed to the interior of the viral capsid, infecting host cells or organisms with the modified viral nucleic acid, and harvesting assembled particles of the modified virus. This procedure is best carried out by direct manipulation of the DNA of the virus in the case of DNA viruses or by manipulation of a cDNA corresponding to the RNA of an RNA virus. Accordingly, in some embodiments, the present invention provides vectors encoding a viral particle that has been modified so as to express an exogenous or foreign peptide on the interior surface of the viral capsid. In particularly preferred embodiments, the nucleic acid sequence encoding a viral coat protein is modified by inserting a sequence encoding an exogenous peptide, so that when the viral coat protein is assembled into a capsid, the exogenous peptide is presented on the interior surface of the capsid. In further

embodiments, the sequence encoding the exogenous peptide is inserted in a portion of the viral coat protein so that assembly of the viral coat protein into a capsid is not substantially interfered with or disrupted.

A wide variety of viral particles find use in the present invention. It is contemplated that both DNA and RNA viruses are suitable for modification be the methods described herein. In particularly preferred embodiments, the modified viral particle is a plant virus. In further preferred embodiments, the plant viruses are preferably icosahedral viruses. To date, all plant viruses with icosahedral symmetry for which crystal structures have been elucidated are characterized by the presence of a canonical eight stranded β-barrel conformation. It is therefore likely that this is a configuration common to all plant icosahedral viruses. Thus, a preferred icosahedral plant virus may be selected from the following virus families: *Caulimoviridae*, *Bromoviridae*, *Comoviridae*, *Geminiviridae*, *Reoviridae*, *Partitiviridae*, *Sequiviridae*, and *Tombusviridae*; and the following virus genera: Luteovirus, Marafiviris, Sobemovirus, Tymovirus, Enamovirus, and Ideavirus.

In particularly preferred embodiments, the modified viral particle is from the family *Comoviridae*. Comoviruses are a group of at least fourteen plant viruses which predominantly infect legumes. Their genomes consist of two molecules of single-stranded, positive-sense RNA of different sizes which are separately encapsidated in isometric particles of approximately 28 nm diameter. The two types of nucleoprotein particles are termed middle (M) and bottom (B) component as a consequence of their behavior in caesium chloride density gradients, the RNAs within the particles being known as M and B RNA, respectively. Both types of particle have an identical protein composition, consisting of 60 copies each of a large (VP37; VP-L) and a small (VP23; VP-S) coat protein. In addition to the nucleoprotein particles, comovirus preparations contain a variable amount of empty (protein-only) capsids which are known as top (T) component.

In the case of the type member of the comovirus group, cowpea mosaic virus (CPMV), it is known that both M and B RNA are polyadenylated and have a small protein (VPg) covalently linked to their 5' terminus. More limited studies on other comoviruses suggest that these features are shared by the RNAs of all members of the group. Both RNAs from CPMV have been sequenced and shown to consist of 3481 (M) and 5889 (B) nucleotides, excluding the poly (A) tails (van Wezenbeek *et al.*, EMBO J. 2:941-46 [1983]; Lomonossoff and Shanks, EMBO J. 2:2253-2258 [1983]). Both RNAs

contain a single, long open reading frame, expression of the viral gene products occurring through the synthesis and subsequent cleavage of large precursor polypeptides. Though both RNAs are required for infection of whole plants, the larger B RNA is capable of independent replication in protoplasts, though no virus particles are produced in this case (Goldbach *et al.*, Nature 286:297-300 [1980]). This observation, coupled with earlier genetic studies, established that the coat proteins are encoded by M RNA.

A 3.5 angstrom electron density map of CPMV shows that there is a clear relationship between CPMV and the T=3 plant viruses such as the tombusviruses, in particular tomato bushy stunt (TBSV) and the sobemoviruses, in particular southern bean mosaic (SBMV). The capsids of these latter viruses are composed of 180 identical coat protein subunits, each consisting of a single β-barrel domain. These can occupy three different positions, A, B and C, within the virions. The two coat proteins of CPMV were shown to consist of three distinct β-barrel domains, two being derived from VP37 and one from VP23. Thus, in common with the T=3 viruses, each CPMV particle is made up of 180 β-barrel structures. The single domain from VP23 occupies a position analogous to that of the A-type subunits of TBSV and SBMV, whereas, the N- and C- terminal domains of VP37 occupy the positions of the C and B type subunits respectively.

X-ray diffraction analysis of crystals of CPMV and another member of the group, bean pod mottle virus (BPMV) shows that the 3-D structures of BPMV and CPMV are very similar and are typical of the comovirus group in general. In the structures of CPMV and BPMV, each β-barrel consists principally of 8 strands of antiparallel β-sheet connected by loops of varying length. The flat β-sheets are named the B,C,D,E,F,G,H and I sheets, and the connecting loops are referred to as the βB-βC, βD-βE, βF-βG and βH-βI loops.

The comoviruses are also structurally related to the animal picornaviruses. The capsids of picornaviruses consist of 60 copies of each of three different coat proteins VP1, VP2 and VP3 each one consisting of a single β-barrel domain. As in the case of comoviruses, these coat proteins are released by cleavage of a precursor polyprotein and are synthesised in the order VP2-VP3-VP1. Comparison of the 3-dimensional structure of CPMV with that of picornaviruses has shown that the N- and C-terminal domains of VP37 are equivalent to VP2 and VP3 respectively and that VP23 are equivalent to VP1. The equivalence between structural position and gene order suggests that VP37 corresponds to an uncleaved form of the two picornavirus capsid proteins, VP2 and VP3.

One of the principal differences between the comoviruses and picornaviruses is that the protein subunits of comoviruses lack the large insertions between the strands of the β-barrels found in picornaviruses though the fundamental architecture of the particles is very similar. The four loops (βB-βC, βD-βE, βF-βG and βH-βI) between the β-sheets are not critical for maintaining the structural integrity of the virions but, in accordance with this invention, are used as sites of expression of foreign peptide sequences, such as antigenic sites from animal viruses.

An advantage of *Comoviridae* is that the capsid contains 60 copies of each of two constituent coat proteins, thereby permitting 60-180 copies of a peptide to be presented per virion wherein the individual coat protein domains have been manipulated such they express inserted peptides. Within the family *Comoviridae*, cowpea mosaic virus and bean pod mottle virus are preferred; of these cowpea mosaic virus is the most preferred.

CPMV is a bipartite RNA virus and in order to manipulate the genome of any RNA virus to express foreign peptides it is desirable to use cDNA clones of the RNA. Accordingly, in some embodiments, the present invention provides cDNA vectors that encode a viral particle modified to express an exogenous peptide on the interior of the viral capsid. Full length cDNA clones of both CPMV RNA molecules are available which can be manipulated to insert oligonucleotide sequences encoding an exogenous peptide. In some particularly preferred embodiments, the vector is CP26. In other embodiments, the vector contains B RNA or M RNA or a variant or homolog of B RNA or M RNA. In some embodiments, the variant or homolog is capble of hybridizing to a plus or minus strand of B RNA or M RNA under conditions of high to low stringency. In particulat preferred embodiments, the variant or homolog contains a sequence encoding an exogenous peptide.

In some embodiments, the cDNA is used to generate *in vitro* transcripts that are infectious when inoculated onto plants. Accordingly, in some embodiments, the present invention provides RNA vectors that encode a viral particle modified to express an exogenous peptide on the interior of the viral capsid. However, the infectivity of the transcripts is significantly lower than that of natural virion RNAs, probably as a result of the presence of non-viral residues at the termini of the transcripts. Difficulties may also be caused by exposure of the transcripts to degradative agents during inoculation. For this reason the transcripts are usually stabilized by capping their 5' ends. In still further preferred embodiments, the modified viral particles also include an exogenous peptide that is presented

on the exterior surface of the viral capsid. Methods for presenting exogenous peptides on the exterior of the viral capsid are provided in U.S. Pat. Nos. 5,874,087 and 5,958,422, each of which incorporated herein by reference.

In further embodiments, the cDNA is used to directly inoculate plants. In these embodiments, the sequences encoding the modified viral particle are operably linked to a promoter that is expressed in plant tissue. Promoters that find use in the present invention include, but are not limited to, the Cauliflower Mosaic Virus (CaMV SD; *see for example*, U.S. Pat. No. 5,352,605, incorporated herein by reference), mannopine synthase, octopine synthase (ocs), superpromoter (*see for example*, WO 95/14098), and *ubi3* (*see for example*, Garbarino and Belknap, Plant Mol. Biol. 24:119-127 [1994]) promoters. This technique overcomes some of the problems encountered with the use of transcripts generated *in vitro* and is applicable to all plant RNA viruses.

In the case of a DNA virus, the DNA itself is introduced into the plant. In this way, the foreign peptide is initially expressed as part of the capsid protein and is thereby produced as part of the whole virus particle. The peptide may thus be produced as a conjugate molecule intended for use as such. Alternatively, the genetic modification of the virus may be designed in order to permit release of the desired peptide by the application of appropriate agents which will effect cleavage from the virus particle.

In order to produce modified virus on a commercial scale, it is not necessary to prepare infective inoculant (DNA or RNA transcript) for each batch of virus production. In some embodiments, an initial inoculant may be used to infect plants and the resulting modified virus may be passaged in the plants to produce whole virus or viral RNA as inoculant for subsequent batches.

In some embodiments, the viral capsid does not contain nucleic acid. Methods are known in the art for the selective enrichment and purification of "empty" virions (*See for example*, van Kammen and de Jaeger, Cowpea Mosaic Virus, In: CMI/AAB Description of Plant Viruses 197, Commonwealth Agricultural Bureax [1978]; and WO 98/56933, the disclosure of which is incorporated herein by reference).

B. Expression of Exogenous Peptides on the Interior of Viral Capsid

One of the limitations of previously described viral expression technologies is the fact that positively charged peptides or hydrophobic peptides inserted in one of the surface loops

of the coat proteins eliminate viral infectivity, due to a disturbed protein folding, particle aggregation, or a disturbed viral transport. The non-viability of these particles is a great hindrance for the expression of some epitopes. Positively charged epitopes can be compensated for by the expression of some additional acidic amino acids (*for example*, pIMM8, pIMM9; Bendalm-nane *et al.*, J. Mol. Biol 290(1):9-20 [1999]). The expression of hydrophobic residues on the surface, however, has so far been very difficult. The use of alternative insertion sites on the virus surface does not solve the problem. The insertion of epitopes in the C-terminus of VP-S, which is on the surface as well, in general gives similar characteristics. This limitation of the technology makes it difficult to express most T-cell epitopes. The discovery of new insertion sites is described below.

1. Insertion Sites

In general, the exogenous RNA or DNA may be inserted into the plant virus genome in a variety of configurations. For example, it may be inserted as an addition to the existing nucleic acid or as a substitution for part of the existing sequence, the choice being determined largely by the structure of the capsid protein and the ease with which additions or replacements can be made without interference with the capacity of the genetically-modified virus to assemble in plants. Determination of the permissible and most appropriate size of addition or deletion for the purposes of this invention may be achieved in each particular case by experiment in the light of the present disclosure. The use of addition inserts appears to offer more flexibility than replacement inserts in some instances.

The present invention demonstrates the insertion of epitopes in viral coat proteins so that they are expressed on the interior surface or side of a viral capsid. In general, any portion of a viral coat protein that is exposed on the interior surface of an assembled viral capsid is a candidate site for epitope insertion. In some embodiments of the present invention, such sites are selected by analysis of high resolution structures (*for example*, crystal structure analysis) of viral capsids. In further embodiments of the present invention, the viral coat protein is modified at the identified site by inserting an epitope. Vectors (*for example*, cDNA or RNA vectors) encoding the modified virus are then used to infect an appropriate host (*for example*, protoplasts, plant tissue, or whole plants). .If infection occurs (*for example*, as assayed by the appearance of local lesions in a plant), then the site is useful for the expression of an epitope. In some instances, serial selection of the infectious virus

identifies mutations leading to greater infectivity, including viral particles capable of systemic infection (discussed in more detail below).

The present invention is exemplified by the insertion of a foreign peptide into VP-S of CMPV. In some embodiments, the site of insertion is the N-terminus of VP-S. In further embodiments, the foreign peptide is inserted at a point between 5 and 20 amino acids from the N-terminus, preferably between 7 and 15 amino acids from the N-terminus, and more preferably between 9 and 12 amino acids from the N-terminus. In preferred embodiments, insertion of the foreign peptide does not perturb the function (*for example*, assembly) of the virus *in vivo*.

The N-terminus is, according to the high resolution structure, on the inside of the virion, rather then on the outer surface. The present invention is not limited to a particular mechanism of action. Indeed, an understanding of the mechanism of action is not necessary to practice the invention. Nevertheless, as a strategy for the expression of epitopes in the N-terminus of VP-S of CPMV, it appears to be desirable to insert the epitope between a duplication of Y11 or V10Y11. The N-terminus plays a role in the viral life cycle, since it is recognized by the viral protease during polyprotein processing. The exact size of the recognition site is not known, but in analogy to the recognition site in between the VP37 and the MP (movement protein) it is probably 10-11 amino acids (Verver *et al.*, Virology 242:22-27 [1998]). The first 10 amino acids of VP-S project from the β-barrel of VP-S, and do not interact with any other residues. Y11 marks the boundary between the N-terminus of VP-S and the rest of the small coat protein and is in a hydrophobic pocket formed by Q73, R165 and H71. Insertion at Y11 apparently does not disrupt polyprotein processing.

Other factors also make this a desirable site. The RNA-distribution inside CPMV is unknown. However, in the middle component of bean pod mottle comovirus, some RNA is observed in the crystal structure. The main RNA-protein interactions take place at the N-terminus of the VP37. This may as well be the case for CPMV. Cryo E.M. pictures of CPMV show that there probably is a small empty space at the five fold symmetry axes, just beneath the protein shell. Furthermore, the N-terminus of VP-S contains two negatively charged residues, which makes an interaction with the sugar-phosphate backbone of the RNA very unlikely as well. Therefore, insertion in the N-terminus of VP-S is unlikely to interfere with RNA interactions and a space exists to accomodate the foreign peptide. Additionally, the N-terminus of VP-S is probably well structured, and the five termini of

symmetry related VP23 molecules in the virion form an annulus (Lin *et al.*, Journal of Virology 74(1): 493-504 [1999]). The B factors indicate that amino acids 1-9 are flexible. Insertions in this region will most likely disturb the annulus, but will probably not affect the folding of the βbarrel. It may be relevant that pepscan experiments indicate that the N-terminus of VP-S in CMPV and in a number of other icosahedral plant viruses represents one of the strongest B-cell epitopes. This is consistent with the notion that this domain (normally buried within the capsid) is temporally exposed through the dynamic behavior (*that is*, "breathing") of the assembled virion such CVPs.

Accordingly, in some embodiments, the present invention provides a modified CPMV having a foreign peptide inserted at Y11. In still further embodiments, the foreign peptide is inserted between a duplication of Y11 of VP-S. In other embodiments, the present invention provides a modified CPMV having a foreign peptide inserted between a duplication of V10Y11 of VP-S. The present invention is not limited to any particular mechanism of action. Indeed, an understanding of the mechanism of action is not necessary to practice the invention. However, it is contemplated that flanking a foreign peptide with a duplication of Y11 or V10Y11 preserves the hydrophobic context of Y11.

The duplication of Y11 represents an engineered modification of the viral vector which has been made to facilitate the accommodation of foreign peptides within the capsid of CPMV. In general, other changes to the amino acid sequence of CPMV have proved difficult to design since many observed *de novo* mutations that occur on particles displaying peptides on the outer aspect of the particle have been limited to changes within the foreign peptide itself.

Several epitopes were successfully inserted at the Y11 position, giving reasonable to very good infection on cowpea plants. Several constructs had a duplication of V10Y11, which in two cases (pNLAL7/pMAL8 and pMV14/pMV15) turned out to be useful to avoid mutations in the epitope or to make the construct infectious. In one construct (pTr4), only V10 was duplicated (because the epitope begins and ends with Val) and also in this case the construct was infectious. These results make it advisable to duplicate at least V10 and if possible V10Y11 in new constructs.

2. Peptides

The foreign peptides which may be incorporated into plant viruses according to this

invention may be of highly diverse types and are subject only to the limitation that the nature and size of the foreign peptide and the site at which it is placed in or on the virus particle do not interfere with the capacity of the modified virus to assemble when cultured *in vitro* or *in vivo*. In broad concept, modified viruses may be formed from any biologically useful peptides (usually polypeptides) the function of which requires a particular conformation for its activity. This may be achieved by association of the peptide with a larger molecule (*for example*, to improve its stability) or mode of presentation in a particular biological system. Examples of such peptides are peptide hormones; enzymes; growth factors; antigens of protozoal, viral, bacterial, or fungal origin; antibodies including anti-idiotypic antibodies; immunoregulators and cytokines (*for example*, interferons and interleukins); receptors; adhesins; and parts or precursors of any of the foregoing types of peptide. The peptide preferably contains more than 5 amino acids.

The present invention allows for the expression of wide variety of foreign peptides on the interior surface of viral capsids. In some embodiments, the peptide is from 5-20 amino acids, preferably from 7-15 amino acids, and most preferably from 8-12 amino acids. However, it is contemplated that the limit on foreign size be limited only by the chimeric virus's capacity to accomodate a foreign peptide and still be capable of assembly into an infectious virus *in planta*. In preferred embodiments, the foreign peptide has immunological properties. Accordingly, in some embodiments, the foreign peptide is an antigen or immunogen. Examples of the epitopes that have been successfully inserted are provided in Table 1 below. In some embodiments, the epitope is a B-cell epitope. In other embodiments, the epitope is a T-cell epitope. In some preferred embodiments, the foreign peptide is a cytotoxic T lymphocyte epitope which is reactive towards cytotoxic T lymphocytes. In general, T-cell epitopes are hydrophobic. In some embodiments, the epitopes have a pI of greater than 7.0 (*for example*, pHBV16, pLCMV2, PVSVI) and are hydrophobic or contain long hydrophobic stretches (*for example*, pLCMV2 and pHBV16). Some of these epitopes had previously been inserted in the βBβC-loop of VP-S, giving good (short HBV epitope), moderate (LCMV epitope), or no (2F10 peptide) symptoms. In some preferred embodiments, the peptide corresponds to SEQ ID NOs: 4-17. In other embodiments, the peptide is encoded by a nucleic acid sequence corresponding to SEQ ID NOs: 18-31.

however, about which residues are optimal in conjunction with certain epitopes. It has been observed that inserting short stretches of alanines on either site of the epitope can be helpful in improving the response to a CTL epitope inserted in a protein carrier (Del Val *et al.*, Cell 66:1145-1153 [1991).

A vector has been made with five alanines in between a duplication of V10Y11, while there is a unique *Not*I site in this insert (=pP35). The vector by itself was infectious an cowpea plants, albeit that the viral symptoms were delayed with respect to WT virus. Inserting epitopes in this oligo-A stretch can be useful to study the optimization of CTL-epitope processing in case epitopes give only weak immune responses. A malaria epitope was inserted in the *Not*I site to make pMAL11. This construct gave good symptoms on plants.

Since no CTL response was observed to the measles virus epitope of pMV15 in mice (see above), an additional construct was made in which this epitope was flanked by several alanines on either side. This construct, pMV16, was not infectious on cowpea plants . In analogy to pSEN3 (see above), a second site mutation from an unrelated construct was applied to pMV16 (MI77V, derived from p'IT4). This construct, which was called pNW17, did not give any symptoms on plants.

The following useful plant viral vectors are on deposit at the American Type Culture Collection (ATCC), Rockville, Md., USA, under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and Regulations thereunder: pTB2 (ATCC No. 75280) and pTBU5 (ATCC 75281). The construction details for these plasmids are set forth in U.S. Patent No. 5,589,367 hereby incorporated by reference.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described compositions and methods of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with particular preferred embodiments, it should be understood that the inventions claimed should not be unduly limited to such specific embodiments. Indeed,

various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art and in fields related thereto are intended to be within the scope of the following claims.

CLAIMS

What is Claimed is:

1. A compound comprising a chimeric viral particle having a capsid, wherein the capsid has an interior side and an exterior side, the capsid comprising at least one exogenous peptide on said interior side of the capsid.

2. A chimeric viral particle according to claim 1 which is capable of assembly in a host cell or tissue.

3. A chimeric virus particle according to claim 1 or 2, in which the virus is icosahedral.

4. A chimeric virus particle according to any of claims 1 to 3, in which the virus is a comovirus.

5. A chimeric virus particle according to claim 4, in which the virus is cowpea mosaic virus.

6. A chimeric virus particle according to any of claims 1 to 5, in which the exogenous peptide is inserted in a coat protein.

7. A chimeric virus particle according to any of claims 1 to 6, in which the exogenous peptide has 5 to 20 amino acids.

8. A chimeric virus particle according to any of claims 1 to 7, in which the exogenous peptide is inserted at a point from 5 to 20 amino acids from the N-terminus of a coat protein such assembly of the viral particle is not precluded in a host cell.

9. A chimeric virus particle according to any of claims 1 to 8, in which the exogenous peptide is inserted in VP-S of cowpea mosaic virus between a tyrosine residue at position 11 and a duplicated tyrosine residue at position 12.

10. A chimeric virus particle according to any of claims 1 to 8, in which the exogenous peptide is inserted in VP-S of cowpea mosaic virus between a dipeptide comprising a valine residue at position 10 and a tyrosine residue at position 11 and a duplicated dipeptide comprising a valine residue at position 12 and a tyrosine residue at position 13.

11. A chimeric virus particle according to any of claims 1-8 in which the exogenous peptide is inserted in VP-S of cowpea mosaic virus between a valine residue at position 10 and a duplicated valine residue at position 11.

12. A chimeric virus particle according to any of claims 1-11, in which the viral particle does not contain nucleic acid.

13. A chimeric virus particle according to any of claims 1-12 in which the exogenous peptide encodes an epitope recognizable by an animal immune system.

14. A chimeric virus particle according to any of claims 1-13, in which the exogenous epitope is a cytotoxic T lymphocyte epitope.

15. A chimeric virus particle according to claims 1-14, in which the exogenous peptide contains a cytotoxic T lymphocyte epitope with flanking amino acids derived from a naturally occurring source of the epitope.

16. A chimeric viral particle according to any of claims 1-12, in which the exogenous peptide is a T helper cell epitope.

17. A chimeric viral particle according to any of claims 1-12 and claim 16, in which the exogenous peptide contains a T helper cell epitope with flanking amino acid sequences derived from a naturally occurring source of the epitope.

18. A chimeric viral particle according to any of claims 1-12, in which the exogenous peptide is a B cell epitope.

19. A chimeric viral particle according to any of claims 1-12 and claim 18, in which the exogenous peptide contains a T helper cell epitope with flanking amino acid sequences derived from a naturally occurring source of the epitope.

20. A chimeric viral particle according to claims 1 to 19, containing a second exogenous peptide expressed on the outer surface of the viral capsid.

21. A chimeric viral particle according to claims 1 to 20, containing a second exogenous peptide expressed on the outer surface of the viral capsid, wherein said peptide is inserted in the βC'-βC" loop of VP-S of cowpea mosaic virus.

22. A chimeric viral particle according to claims 1 to 20, containing a second exogenous peptide expressed on the outer surface of the viral capsid, wherein said peptide is inserted in the βC-βC loop of VP-S of cowpea mosaic virus.

23. A chimeric viral particle according to claims 1 to 20, containing a second exogenous peptide expressed on the outer surface of the viral capsid, wherein said peptide is inserted in the βE-βA loop of VP-L of cowpea mosaic virus.

FIG. 1

Insertions in the N-terminus of CPMV VP-S

pCP26:

CAAGGACCTGTTTGTGCTGAAGCCTCAGATGTGTATAGCCCATGTATGATAGCTAGCACTCCTCCTGC

GTTCCTGGACAAACACGACTTCGGAGTCTACACATATCGGGTACATACTATCGATCGTGAGGAGGACG

Q G P V C A E A S D V Y S P C M I A S T P P A

With NheI/Eco0109I:

CAAG

GTTCCTG

CTAGCACTCCTCCTGC

GTGAGGAGGACG

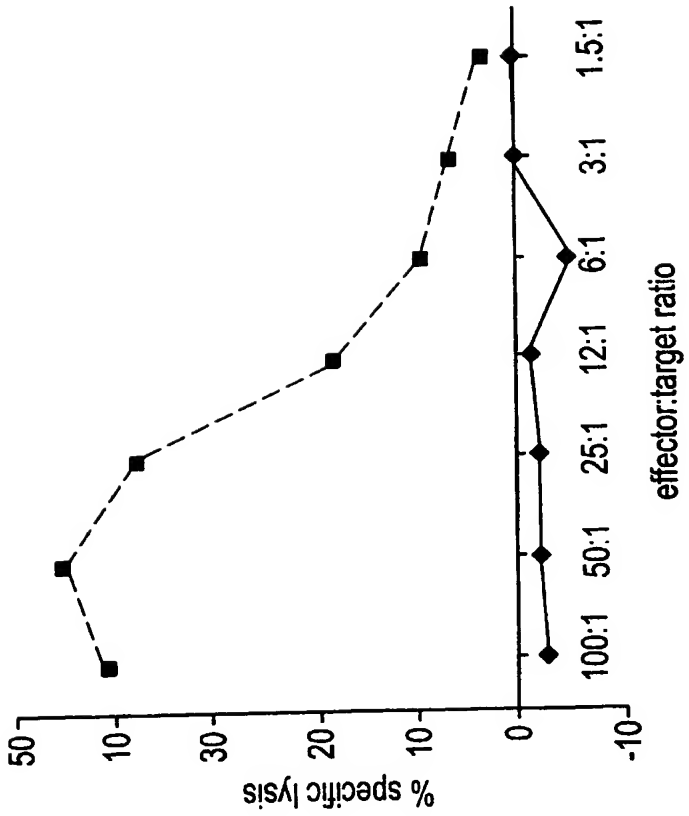
Inserts:

GACCTGTTTGTGCTGAAGCCTCAGATGTGTAT -epitope- TATAGCCCATGTATGATAG

GACAAACACGACTTCGGAGTCTACACATA ATATCGGGTACATACTATCGATC

duplication of y_{11}

FIG. 2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10
30
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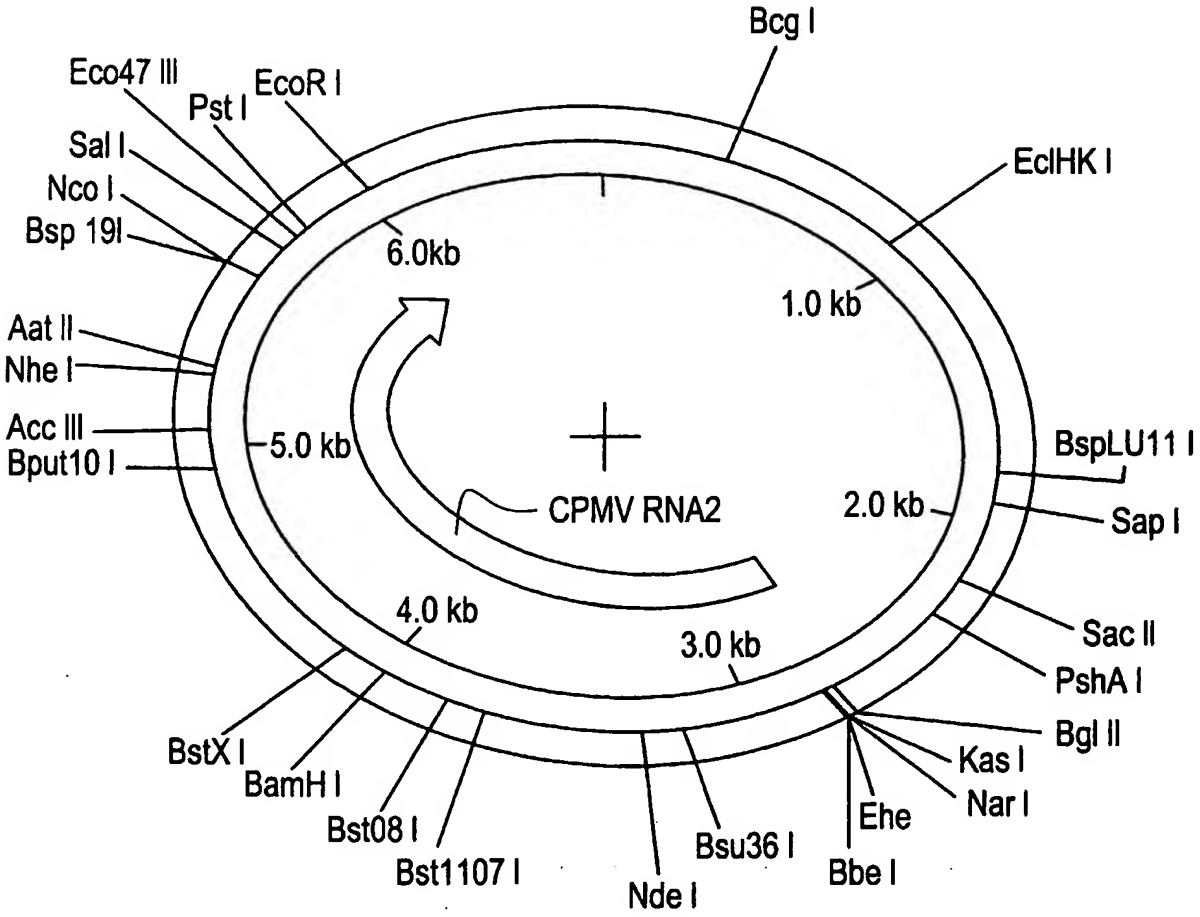
3:1
1.5:1
effector:target ratio

FIG. 3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1.0 kb
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Nhe I
Acc III
Bput10 I
5.0 kb
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BspLU11 I
2.0 kb
Sap I
4.0 kb
3.0 kb
Sac II
PshA I
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INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 00/28430

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/40 C12N15/62 C12N7/01 C07K14/08 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CASAL J.I.: "Use of parvovirus-like particles for vaccination and induction of multiple immune responces" BIOTECHNOL. APPL. BIOCHEM., vol. 29, April 1999 (1999-04), pages 141-150, XP002162594 the whole document page 148; figure 2	1-3,6-8, 12-20
Y	see above	4,5, 9-11, 21-23

Y	WO 92 18618 A (AGRICULTURAL GENETICS CO ;PURDUE RESEARCH FOUNDATION (US)) 29 October 1992 (1992-10-29) the whole document page 5, second paragraph	4,5, 9-11, 21-23

	-/--	

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Celler, J

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 56933 A (JOHN INNES CENTRE ;LOMONOSSOFF GEORGE PETER (GB); TAYLOR KATHRYN M) 17 December 1998 (1998-12-17) the whole document	1-23

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INTERNATIONAL SEARCH REPORT

Information on patent family members

national Application No
PCT/US 00/28430

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9218618 A	29-10-1992	AT 154635 T	15-07-1997
		AU 661479 B	27-07-1995
		AU 1447992 A	17-11-1992
		CA 2108777 A	20-10-1992
		DE 69220485 D	24-07-1997
		DE 69220485 T	09-10-1997
		DK 580635 T	19-01-1998
		EP 0580635 A	02-02-1994
		ES 2106173 T	01-11-1997
		GR 3024739 T	31-12-1997
		HU 65554 A,B	28-06-1994
		JP 6506583 T	28-07-1994
		NZ 242430 A	25-06-1993
		US 6110466 A	29-08-2000
		US 5874087 A	23-02-1999
		ZA 9202604 A	30-12-1992
WO 9856933 A	17-12-1998	AU 8030098 A	30-12-1998

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